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Defective and nondefective adenovirus vectors for expressing foreign genes in vitro and in vivo

(Recombinant virus; major late promoter; gene expression)

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SUMMARY

We have constructed recombinant adenoviruses (Ad), with functional or defective *E1a* genes, which harbor either the hepatitis B (HB) virus *s* gene encoding the HB surface antigen, as well as the pre-S2 epitopes, or the bacterial gene encoding chloramphenicol acetyltransferase (CAT) under control of the Ad major late promoter (MLP). The recombinant viruses defective for *E1a* (Ad.MLP.S2 and Ad.CAT), which can be efficiently propagated only on 293 cells that complement this defect, and the nondefective (Ad.MLP.S2.E1A) recombinant were used to infect a wide spectrum of cells of different origin. The yields of HBs and CAT proteins obtained with these different recombinant viruses demonstrate no real advantage to using nondefective vectors, whatever the cell type infected. The injection into chimpanzees of Ad.MLP.S2 does not elicit the production of antibodies, but can immunologically prime the animals, resulting in a partial protection against HBV challenge.

INTRODUCTION

The human adenovirus (Ad) presents several advantages which can render it a generally useful vector to express foreign genes (Ballay et al., 1985).

In this study, we describe the construction of *E1a*-defective and nondefective recombinant adenoviruses, and compare the efficiencies of expression of the cloned genes in cell culture (i.e., the HBV middle envelope protein gene, including the *s* and the pre-*s2* regions of the *s* gene, and the bacterial reporter gene *cat*). These recombinants make use of the adenovirus major late promoter and a nearly complete copy of the tripartite leader sequence to express the foreign genes (Chow et al., 1977; Lewis et al., 1984). When injected into rabbits, Ad.MLP.S2 elicits the production of antibodies against the middle and major envelope proteins. However, only one out of the two chimpanzees inoculated with this same recombinant was fully protected against hepatitis following a challenge with HBV, while the other experienced a modified HBV infection.

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Abbreviations: Ad, adenovirus; Ag, antigen; anti-HBc, antibodies against HB core Ag; anti-HBs, antibodies against HB surface Ag; bp, base pair(s); CAT, chloramphenicol acetyltransferase; *cat*, gene encoding CAT; *E1a*, gene encoding E1A; *E1b*, gene encoding E1B; HB, hepatitis B; HBV, HB virus; ID, infectious dose; I.U., international unit(s); kb, kilobase(s) or 1000 bp; MLP, major late promoter; moi, multiplicity of infection; m.u., map unit(s); nt, nucleotide(s); p.i., post infection; P/N, positive/negative; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; pfu, plaque-forming unit(s); pre-S2, middle envelope protein of HBV; pre-*s2*, pre-S2-encoding region; RIA, radioimmunoassay; *s*, gene encoding HBsAg; S, major envelope protein of HBV; SV40, simian virus 40; wt, wild type.

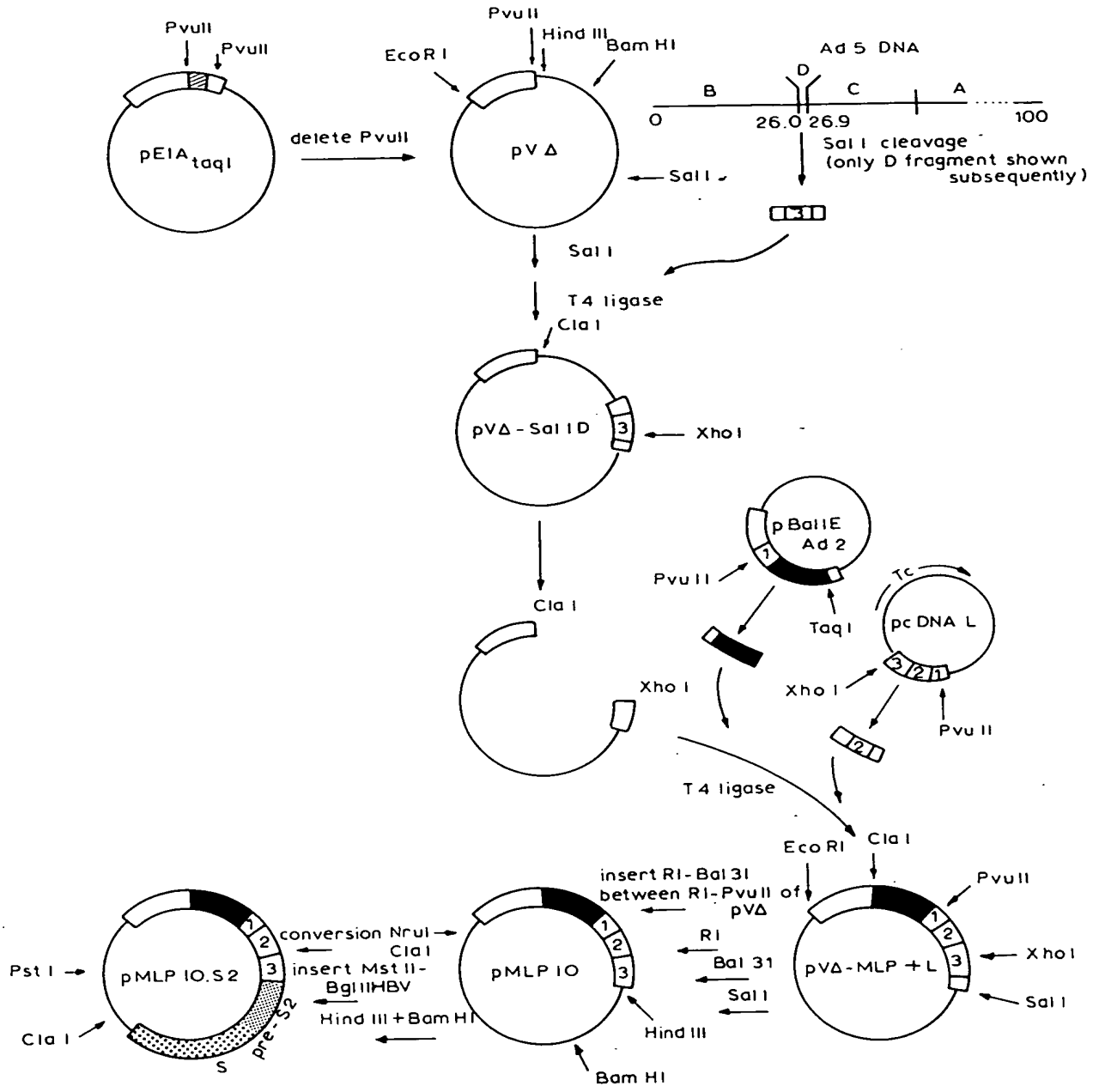

Fig. 1. Construction of plasmid pMLP10.S2. The structure of $pE1A_{taqI}$ has been presented elsewhere (Ballay et al., 1985). Essentially, it retains the leftmost 631 bp of Ad5. Plasmid pVΔ was obtained by deletion of the *E1a* sequences (nt 455–625) using *Pvu*II. The adenoviral DNA fragment *Sal*I-D (nt 9455–9835), which contains the entire third leader was inserted into the *Sal*I site of pVΔ (plasmid pVΔ-SalID). Plasmid pcDNAL, containing the leader sequences derived from an Ad2 cDNA clone, was digested with *Xho*I + *Pvu*II while pBallEAd2 (14.7–21.5 m.u.), which contains the MLP, was digested with *Taq*I + *Pvu*II. The fragments of interest were then inserted between the *Cla*I and *Xho*I sites of pVΔ-*Sal*I-D. The resultant plasmid, pVΔ – MLP + L, contains the Ad2 MLP and the intact leader sequences, flanked on their left by the leftmost 455 bp of Ad5 viral genome. This plasmid was linearized by *Sal*I and mildly digested with exonuclease BAL 31 to remove the splice donor site located at the end of the third leader. *Eco*RI BAL 31 fragments were inserted between the *Eco*RI and *Pvu*II sites of pVΔ. One clone named pMLP10 was found to retain the first and second Ad2 leaders and 80 out of the 90 nt of the third leader. Plasmid pMLP10.S2 was constructed by inserting the fragment *Mst*II-*Bgl*II (nt 3161–1982) of the HBV genome coding for the HBsAg as well as for the pre-S2 epitopes between the *Hin*dIII and the *Bam*HI sites of pMLP10 after filling-in the ends. The *Mst*II site precedes the start codon of the pre-s2 region by 9 nt, whereas the *Bgl*II site is located 64 nt downstream from the poly(A)-addition signal of the *s* gene. Open boxes, sequences for Ad left end; blackened boxes, MLP from Ad; open boxes marked 1, 2 and 3, Ad leader sequences; stippled boxes, HBV sequence; thin line, plasmid sequence.

RESULTS AND DISCUSSION

(a) Construction of the adenovirus HBV recombinants

The Ad5 recombinants Ad.MLP.S2, with most of the *E1a* region substituted by a chimeric gene, and Ad.MLP.S2.E1A, similar to the former but containing a functional *E1a* gene, were constructed according to the protocol previously described (Ballay et al., 1985). To construct Ad.MLP.S2, a short DNA fragment containing a *Cla*I site was inserted into the *Nru*I site of plasmid pMLP10.S2 (Fig. 1) to allow the ligation of the pMLP 10.S2 *Pst*I-*Cla*I fragment to the large *Cla*I fragment of Ad5dl327 DNA (2.6–100 m.u.). In the case of Ad.MLP. S2.E1A, the *Pst*I-*Cla*I fragment of pMLP.S2.E1A (Fig. 2) was directly ligated to the large *Cla*I fragment of Ad5 dl327.

(b) Construction of the adenovirus *cat* recombinants

Recombinant plasmids pMLP.CAT, pMLP.CAT.E1A, and pMLP.CAT.inv. were used to generate corresponding viruses Ad.CAT, Ad.CAT.E1A, and Ad.CAT.inv. (Fig. 3). In Ad.CAT and Ad.CAT.E1A, the DNA segment encoding the CAT protein is positioned downstream from a series of sequences: the Ad5 left terminus (nt 1–455 of Ad5), the Ad2 MLP, a cDNA sequence encoding the Ad2 tripartite leader; it is followed by splicing (nt 4705–4099) and polyadenylation (nt 2770–2553) signals from SV40. Furthermore, the ligation allowed creation of a full copy of the *E1a* gene. In the case of the Ad.CAT.inv., the hybrid transcription unit is also located downstream from the Ad5 left terminus, but in a reverse orientation with respect to viral replication sense.

(c) Growth characteristics and host dependence for viral replication of the adenovirus recombinants

To compare the growth of the Ad.MLP.S2 and Ad.MLP.S2.E1A recombinants in 293 cells and in human cells not transformed by Ad5 (HeLa and HepG2 cells), virus stocks were titrated on these three cell types in a plaque assay. Equivalent titers were found for wt strain and both recombinants on 293 cells. The defective recombinant Ad.MLP.S2 produced no detectable plaques on HeLa cells, and only a small number of plaques on HepG2 cells. Stocks of the Ad.MLP.S2.E1A virus on HeLa or HepG2 cells were comparable to those obtained on 293 cells, thus demonstrating this virus' ability to behave, at least in vitro, as a nondefective, fully competent, wt adenovirus.

With regard to the *cat* recombinant viruses, each one replicates to levels equivalent to Ad5dl327 in 293 cells. However, plating efficiency of Ad.CAT.E1A was identical on 293 and Vero cells, showing the restoration of a functional *E1a* gene for the latter virus.

(d) HBsAg and CAT activity expressed by the recombinant adenoviruses

The infection of 293 and Vero cells by Ad.MLP.S2 at a moi of 10 pfu per cell (as determined by titration on 293 cells) leads to an accumulation in the medium of HBsAg of 6 $\mu g/10^6$ cells and of 11 $\mu g/10^6$ cells, respectively (Fig. 4A). HBsAg particles showed a density of 1.2 g/cm^3 and carry both middle and major HBV envelope proteins (Fig. 4B).

In view of the usefulness of a eukaryotic vector able to express foreign genes at high levels in a wide spectrum of mammalian cells, we tested our recombinant in several cell lines of different species and embryological origins

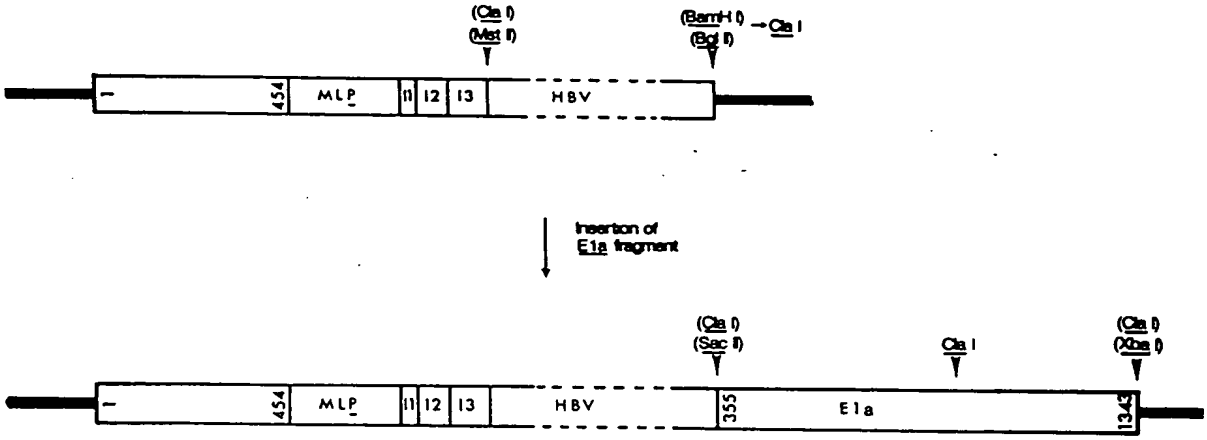

Fig. 2. Construction of plasmid pMLP.S2.E1A. To construct the plasmid pMLP.S2.E1A, the *Cla*I site of pMLP10.S2, created at the end of the HBV sequences by ligation of the PolIk-filled ends of *Bgl*II and *Bam*HI, has been used to insert the adenovirus fragment *Sac*II-*Xba*I (nt 353–1340). Thick lines correspond to pBR322 sequences while the cloned nt sequenees are boxed. HBV genes, not drawn to scale, are represented as dashed lines.

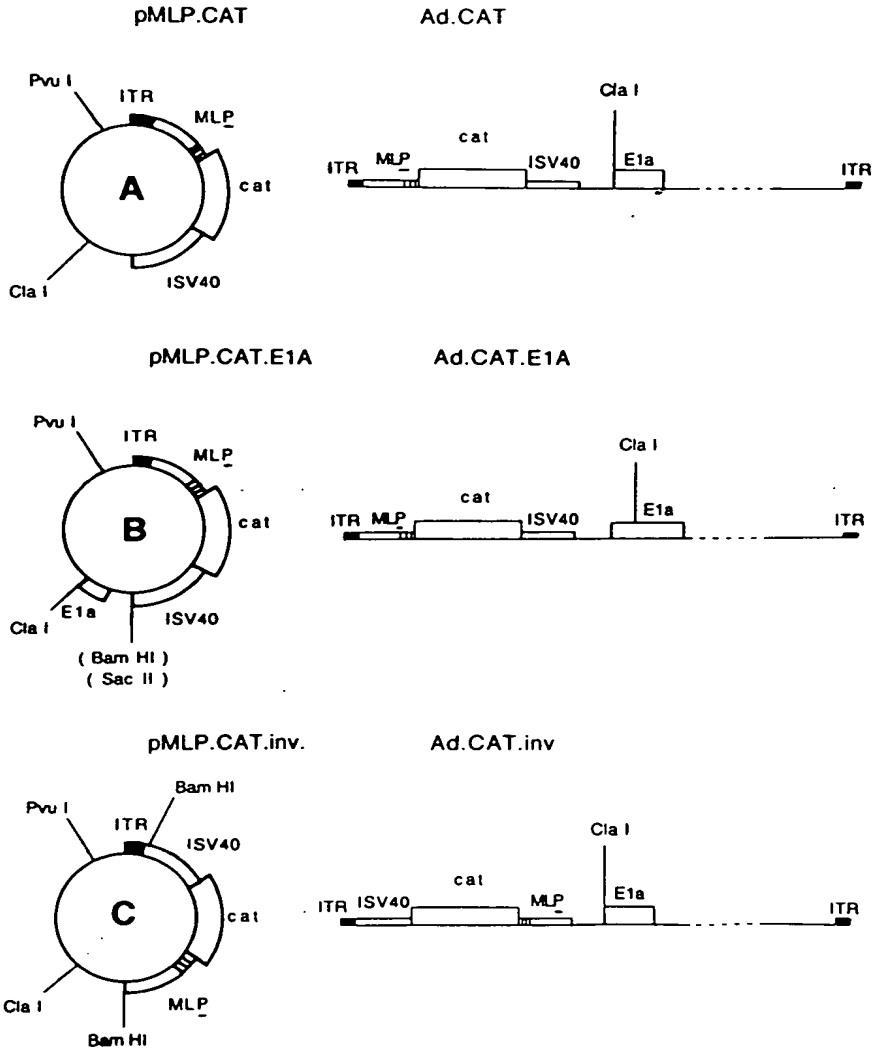

Fig. 3. Construction of Ad.CAT recombinant viruses from plasmids pMLP.CAT (plasmid A), pMLP.CAT.E1A (plasmid B) and pMLP.CAT.inv. (plasmid C). Plasmid pMLP.CAT has been obtained by inserting between the *Hin*dIII and *Bam*HI sites of pMLP10, the *Hin*dIII-*Bam*HI fragment from pSV.CA$^-$ containing the *cat* region followed by the SV40 intron and polyadenylation sequences. A *Cla*I site was subsequently inserted into the *Nru*I site pMLP.CAT to construct the recombinant adenovirus following the same way reported for plasmid pMLP10.S2. Plasmid pMLP.CAT.E1A was constructed by insertion of a *Sac*II-*Cla*I fragment, containing nt 353–917 from the left end of Ad5 [except for nt 916, changed to C (underlined), and nt 923 changed to T (underlined), using the synthetic oligo 5′–GGCAGGTAACATCGATTACCTCCGG, and a mutagenesis kit supplied by Amersham] between the unique *Bam*HI (filled with PolIk) and *Cla*I sites of pMLP.CAT. Thus, this plasmid contains the 5′-terminal part of the *E1a* gene, downstream from the *cat* gene and in the same orientation. In regard to plasmid pMLP.CAT.inv., a 117-bp *Sac*II fragment (generated from the *Sac*II sites located at nt 353 and 5794 in the genome of Ad2) was excised from pMLP.CAT and replaced by a synthetic linker *Sac*II-*Bam*HI-*Sac*II. This modified plasmid was fully cleaved with *Bam*HI and then self-ligated to obtain recombinants containing the hybrid transcription unit (Ad.MLP plus tripartite leader plus *cat* gene) in a reverse orientation. Plasmids were digested with *Pvu*II + *Cla*I, ligated to the large *Cla*I fragment of Ad5dl327 and the ligation mixture was used to transfect 293 cells (Graham and Van der Eb, 1973). ITR, inverted terminal repeat of Ad; MLP, major late promoter and tripartite leaders; i SV40, intervening sequence of SV40.

(Table I). The defective Ad.MLP.S2 drove the synthesis of substantial, although variable, amounts of HBsAg in all the cell lines tested. These levels were usually higher than those obtained with the nondefective Ad.MLP.S2.E1A recombinant in all the cell lines but those of lymphoid origin. Under the conditions used in these experiments, the rapid onset of a lytic infection with the nondefective recombinant may account, at least in part, for this phenomenon. In cells of lymphoid origin, it has been demonstrated that, even in the presence of fully competent wt adenovirus, several steps in the adenovirus replicative cycle are not efficient (Silver and Anderson, 1988). In this context, the combined effects

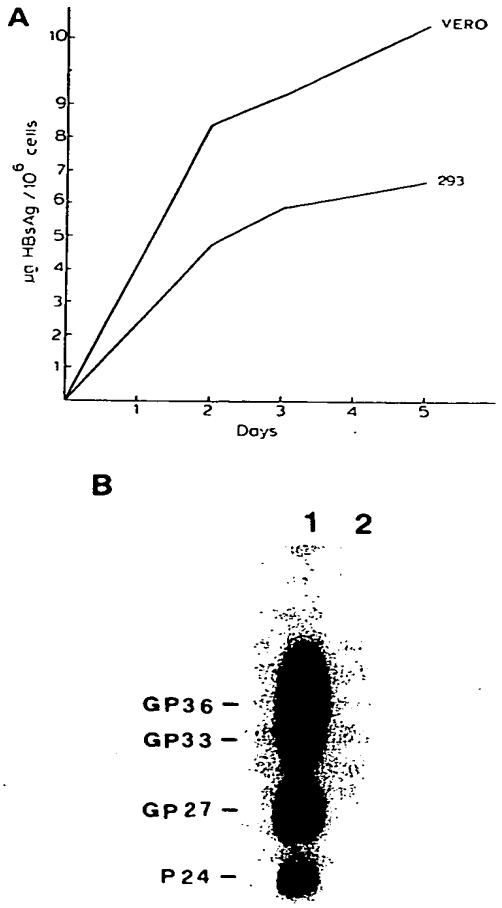

Fig. 4. Adenovirus-mediated HBs production. (Panel A) Cumulative amounts of extracellular HBsAg (expressed in µg) produced after infection of 10^6 293 cells and 10^6 Vero cells by the Ad.MLP.S2 recombinant. HBsAg was detected by RIA (AUSRIA II, Abbott Labs.), and quantified using a parallel line assay with an HBsAg standard (20 ng/ml). (Panel B) Autoradiograph of ^{35}S-labeled polypeptide material from cell-culture medium immunoprecipitated with rabbit anti-HBs antiserum. 48 h after transfection, cells were grown for 1 h in methionine-free medium and radiolabeled for 3 h with 150 µCi/ml of [^{35}S]methionine (1064 Ci/mmol, New England Nuclear) per dish. Medium was clarified by centrifugation for 1 h at 650 × *g* and concentrated to 50 µl using a Centricon 10 micro-concentrator (Amicon). Immunoprecipitation was performed as described previously (Weimer et al., 1987) with all the concentrated medium, and 5 µl of antiserum. Immunoprecipitated proteins were analyzed by 0.1% SDS–PAGE following the Laemmli (1970) procedure. After electrophoresis, the gel was soaked for 1 h in a solution of 30% methanol/10% acetic acid, treated with Enhance (New England Nuclear) as indicated by the supplier, dried and exposed to an x-ray film at −70°C. Lanes: 1, 293 cells infected by the Ad.MLP.S2 recombinant; 2, 293 cells infected by wt Ad5. P24 and GP27 represent the 24-kDa nonglycosylated and the 27-kDa glycosylated major envelope proteins. GP33 and GP36 represent the middle envelope proteins glycosylated in the pre-S2 region or in both the pre-S2 and the S regions, respectively.

TABLE I

Levels of expression of HBsAg after infection with the recombinant adenovirus

Cell lines[a]	Recombinant adenovirus	
	Ad.MLP.S2	Ad.MLP.S2.E1A
	Levels of HBsAg (ng/10^6 cells)[b]	
Human		
293	6000	3900
HeLa	1540	1150
HepG2	1850	1430
Raji	450	2150
EBV lymphoblastoid	1050	1780
Simian		
Vero	11600	5430
Mouse		
NIH3T3	850	780
L	430	170
Rabbit		
RK13	950	860
537	480	250

[a] 293 (Harrisson et al., 1977); HeLa (Gey et al., 1952); HepG2 (Knowles et al., 1980); Raji (Pulvertaft, 1964); NIH3T3 (Jainchill et al., 1969); L (Sanford et al., 1948); RK13 (Beale et al., 1963).

[b] Cells were infected at an moi of 10 pfu per cell; supernatants were collected 120 h p.i. and tested for HBsAg reactivity as described (Ballay et al., 1985).

of the upstream *E1a* enhancer sequence, of the downstream HBV enhancer (which has been reported to work efficiently in lymphoid cells) (Elfassi, 1987), and of the *E1a* gene product, contribute to the high rate of transcription from the Ad MLP. The level of HBsAg and of *cat* expression observed in Vero cells and in other non-Ad5-transformed cells is very high despite the fact that the Ad MLP is known to be tightly controlled throughout the viral infectious cycle. However, it has been demonstrated that by culturing Ad-infected HeLa cells for prolonged periods in the presence of an inhibitor of viral replication, not only is there an important production of *E1a* and *E1b* early gene mRNAs along with their corresponding proteins, but the late viral protein hexon, is also accumulated (Gaynor et al., 1982).

Summarized in Fig. 5A are the results dealing with the intracellular accumulation of CAT, upon infection of either 293 or Vero cells by Ad.CAT, which confirm those obtained with the HBV recombinants. In 293 cells, maximal accumulation occurs 48 h p.i. Then, a rapid decrease in CAT activity takes place, correlated with the strong cytopathic effects observed (Fig. 5A). Interestingly, the presence of a viral copy of the *E1a* gene does not alter *cat* expression. Neither is there a difference in CAT levels with Ad.CAT and Ad.CAT.E1A in 293-infected cells, or a difference between 293 and Vero cells infected with Ad.CAT.E1A. However, a displacement in the activity curve is observed with

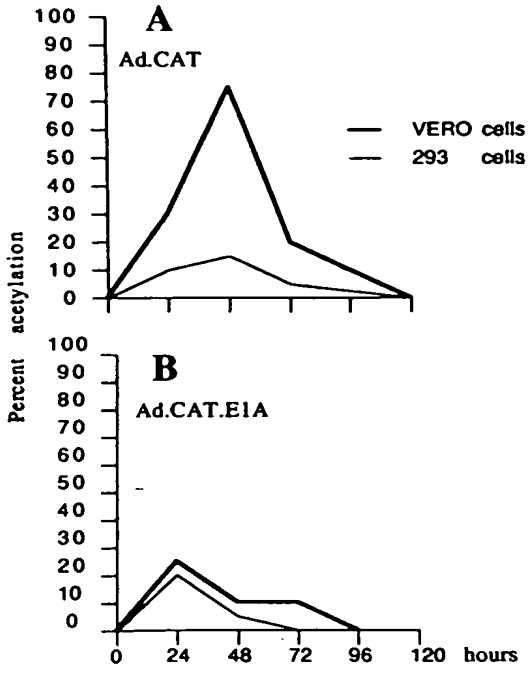

Fig. 5. Kinetics of CAT accumulation following infection with either Ad.CAT (A) or Ad.CAT.E1A (B). The 293 and Vero cells were infected at an moi of 10 pfu/cell and harvested every 24 h. CAT transient assays were performed as described by Gorman et al. (1982). The % acetylation is given for 0.5 μg of total proteins being tested.

Ad.CAT.E1A (Fig. 5B). The maximum CAT level occurs 24 h p.i. instead of 48 h. We propose, as an explanation, that *cis*-acting functions are brought by the *E1a* gene and facilitate the transition from the early to the late step of viral replication. Alternatively, the E1A could be synthesized at higher levels when the *E1a* gene is brought by the viral genome and would thus facilitate this transition.

To investigate the role of the *E1a* enhancer sequence in our recombinants, we have constructed the Ad.CAT.inv. defective virus, containing the cat hybrid transcription unit in a reverse orientation with regard to the *E1a* enhancer sequence. Maximum CAT levels are summarized in Table II. There is no significant difference between Ad.CAT or Ad.CAT.E1A, and Ad.CAT.inv. in 293 cells, compared to Vero cells where the maximum CAT level is about ten to 100 times higher when the *E1a* enhancer is adjacent to the MLP.

Irrespective of the mechanism involved, our results indicate that adenovirus can be a generally useful vector to express foreign genes at high levels in a wide spectrum of cells.

(e) Biological activity of Ad.MLP.S2 in rabbits and chimpanzees

To test the ability of the adenovirus recombinant Ad.MLP.S2 to direct the synthesis in vivo of HBsAg particles carrying pre-S2 epitopes, rabbits were inoculated i.v. with highly purified preparations of either recombinant or wt Ad5. Antibodies directed against both middle and m: HBV envelope proteins could easily be detected as of the first week following the injection, as we have already reported (Ballay et al., 1985; data not shown).

TABLE II

Comparison of maximum CAT accumulation levels following infection with recombinant adenovirus, in 293 cells and Vero cells

Virus[a]	Cell lines[b]	
	293	Vero
Ad.CAT	17	77
Ad.CAT.E1A	20	23
Ad.CAT.inv	20	< 5

[a] Infections were performed at an moi of 10 pfu per cell in every case. Values correspond to the % of acetylation, calculated as described and corrected for 0.5 μg of total proteins.
[b] For references, see Table I, footnote a.

The ability of the recombinant Ad.MLP.S2 virus to protect against hepatitis was then tested in two chimpanzees which were vaccinated twice with a three-month interval by i.v. administration of 10^9 pfu of the recombinant per animal (Fig. 6). Neither of the animals developed side effects during the vaccination period. All sera were negative for elevations in liver enzyme activity and for evidence of exposure to HBV antigens. The control chimpanzee 2026 (Fig. 6) that had not received the adeno-HBV recombinant, developed a typical B-type hepatitis. HBsAg appeared eleven weeks after the HBV challenge and reached a P/N ratio value of 43. Anti-HBc and biochemical evidence of hepatitis were detected after 18 and 20 weeks, respectively. HBsAg levels declined and disappeared 30 weeks after challenge, coincident to the appearance of anti-HBs antibodies. Even in the absence of circulating anti-HBs, the chimpanzee Oscar was immunologically primed and the rapid and sustained antibody response after challenge with live HBV is in keeping with this hypothesis. The same phenomenon of protection after challenge even in the absence of seroconversion to anti-HBs was also described by Moss et al. (1984) in chimpanzees vaccinated by a recombinant vaccinia virus.

The chimpanzee Oscar, which received the recombinant adenovirus, showed no detectable HBsAg or biochemical evidence of hepatitis. Six weeks after the challenge, anti-HBs appeared and reached high levels that persisted during the rest of the experiment. On the contrary, the second animal, Theo, developed a mild case of hepatitis characterized by the appearance of HBsAg 14 weeks after the challenge, followed by a slight elevation of alanine amino-

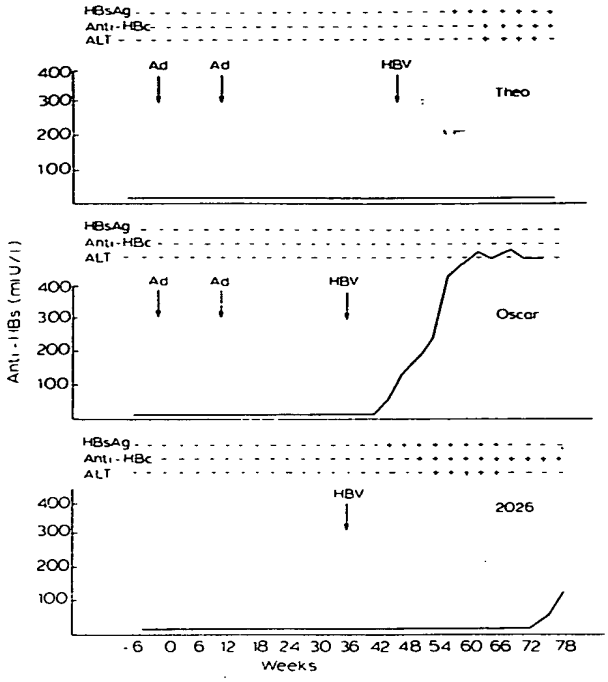

Fig. 6. Chimpanzee vaccination. Three chimpanzees, sero-negative for HBV markers, as determined with commercially available RIA (HBsAg; AUSRIA; anti-HBs; AUSAB; anti-HBc; CORAB; antiHBc IgM; CORZYME M; Abbott Laboratories), were used. None of the animals showed evidence of hepatitis as judged by normal levels of alanine aminotransferase and aspartate aminotransferase. In the animals named Oscar and Theo, 1 ml containing 10^9 pfu of the recombinant virus was injected and a booster was performed twelve weeks later. The recombinant adenovirus stock was free of any contamination by HBsAg particles as judged by RIA and electron microscopy. The third chimpanzee, numbered 2026, did not receive the vaccine, and served as a control. The three chimpanzees were subsequently challenged i.v. with $10^{3.5}$ chimpanzee ID_{50} units of live HBV (subtype ayw, strain MS-2). Oscar and Theo received HBV, 24 weeks and 36 weeks, respectively, after the booster inoculation. Such a dose and strain have been demonstrated to consistently induce HB in susceptible chimpanzees (Barker et al., 1975). Serum samples were obtained at weekly intervals after the HBV challenge. Alanine aminotransferase (ALT) activity is expressed in I.U./liter and levels exceeding twice the mean of the baseline are considered high and indicated by the symbol +. Anti-HBs antibodies are expressed as the ratio of sample c.p.m.: negative control c.p.m. (S/N). Positive HBsAg values (2.1 P/N) and positivity for anti-HBc antibodies are indicated by the symbol +.

transferase levels. Thus, the chimpanzee Theo developed both serological evidence of HBV infection and biochemical evidence of liver disease in spite of an anterior inoculation with the recombinant adenovirus. Similar results, i.e., full protection of one animal and modified HBV-induced disease in another, have recently been obtained using an oral HB vaccine based on a live recombinant adenovirus (Lubeck et al., 1989).

Although additional information is needed concerning the efficacy to induce a protective immune response in animals, adenovirus constitutes a useful model towards the development of live vaccines. In this regard, a vaccine containing live infectious adenovirus in an enteric-coated dosage form is already marketed, and has proven to be effective and at the same time free of significant side effects (Chanock et al., 1966; Edmonson et al., 1966). Moreover, the release of adenovirus into the intestine is followed by its replication there without causing adenoviral disease, yet inducing the formation of adenovirus antibodies that render adenoviral recombinants suitable candidates for the development of vaccines against enteric infections.

(f) Conclusions

(1) No real advantage in using nondefective vectors for the production in cell cultures of foreign proteins can be demonstrated.

(2) The HBsAg synthesized by the recombinant adenovirus Ad.MLP.S2 is similar, if not identical, to material from the serum of human HBV carriers. The HBsAg was excreted as 22-nm particles carrying pre-S2-encoded determinants.

(3) The preliminary results from chimpanzees receiving i.v. the Ad.MLP.S2 recombinant show that only one out of two injected animals was protected against a challenge with HBV, the second animal being insufficiently protected by the vaccination. The injection of chimpanzees with HB vaccines generally constitutes a safety test, however, the potential of our adenovirus recombinant as a live vaccine is being assessed by oral administration since this would be the preferred route of vaccination.

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